The Impact of environmental enrichment on the murine inflammatory immune response

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Conflict of Interest statement

The authors have declared that no conflict of interest exists.

Abstract

Living in a mentally and physically stimulating environment has been suggested to have a beneficial effect on the immune response. This study investigates these effects, utilizing a two-week program of environmental enrichment and two models of acute inflammation: zymosan-induced peritonitis (ZIP) and the caecal ligation and puncture (CLP) model of sepsis. Our results revealed that following exposure to an enriched environment (EE) mice possessed significantly higher circulating neutrophil to lymphocyte ratio compared to control animals. When subject to ZIP, EE animals exhibit enhanced neutrophil and macrophage influx into their peritoneal cavity. Corresponding results were found in CLP where we observed an improved capacity for enriched animals to clear systemic microbial infection. Ex Vivo investigation of leukocyte activity also revealed macrophages from EE mice presented an enhanced phagocytic capacity. Supporting these findings micro-array analysis of EE animals revealed the increased expression of immune-modulatory genes associated with a heightened and immune-protective status. Together these results provide novel mechanisms by which environmental enrichment influences the development and dynamics of the immune response.

Introduction

Inflammation plays a pivotal role in the normal function of many living organisms serving both as an initiator of the immune response and a key modulator of tissue homeostasis [1]. Activated by a diversity of internal and external stimuli, inflammation aids in the clearance of pathogens, dead tissue and foreign bodies as well as re-modelling tissues to facilitate re-growth and repair.

Inflammation is distinct from other homeostatic mechanisms in that damage to self is an unavoidable and often necessary part of its process. As such, uncontrolled inflammation can be highly detrimental leading to irreparable tissue damage and potentially, autoimmune disease. While a long studied field of research [2], recent innovations in cellular and molecular biology have greatly expanded our knowledge of the factors regulating (and dysregulating) the inflammatory response.

Such knowledge has allowed us to divide this process into several subtypes such as; silent inflammation (due to ageing or lifestyle), sterile inflammation (e.g. gouty arthritis) [3], para-inflammation (e.g. macular degeneration) [4] and cellular stress responses [5], each with its own distinct effects and aetiologies. Accordingly, we now appreciate that inflammation is a multifaceted and adaptive process, sensitive to alterations both internal and external to the host.

A growing number of studies have identified that non-heritable or environmental factors play a significantly greater role influencing the inflammatory response than previously recognised [6]. Multiple inflammatory and autoimmune diseases have been linked to circumstances such as diet [7], psychological state [8], living conditions [9] and socioeconomic status [10]. While the growing availability of clinical and epidemiological data provides us with a broad picture of the incidence and outcomes of this cross-

66 talk [11,12], there is a strong need for further research into the mechanisms by which
67 these factors achieve this influence.

68 Corresponding animal research in the field of neurology has utilised environmental
69 enrichment (EE) - an experimental paradigm that attempts to model an active social,
70 mental and physical life in captive animals [13]. Such research has revealed how a
71 common feature of EE is its ability to bring about an improved capacity to return to a
72 normal homeostatic state. For example, exposure to EE has been shown to mitigate
73 or facilitate repair of neurological damage caused by chemical exposure [14], physical
74 trauma [15] and neurodegenerative disease [16].

75 Alongside these neurological effects a small number of investigations have reported
76 the modulating effects EE has upon the immune response. For example one study
77 demonstrated that following four months in an enriched environment, mice show an
78 improved response to infection with influenza type A, clearing the virus more quickly
79 than animals housed in a standard environment [17]. Further studies on mice have
80 also suggested that EE may have specific immune modulatory effects, enhancing
81 leukocyte effector function while [13] decreasing levels of circulating inflammatory
82 cytokines in circulation [18,19]. Such an unusual immune profile would appear likely to
83 promote an equally unique inflammatory response. However, as of yet no direct
84 research has investigated the effects of environmental enrichment on classical
85 experimental models of inflammatory disease.

86 We applied an established enrichment paradigm alongside classic experimental
87 models of disease, exposing groups of male outbred CD1 mice to a 14-day
88 environmental enrichment regime then assessing the scale and intensity of their
89 immune response in the context of experimental sepsis or peritonitis. Our results

revealed environmental enrichment has an enhancing effect on the murine immune inflammatory response. We found that housing in an enriched environment induced better clearance of systemic infection in a model sepsis without affecting the production of inflammatory markers. These effects are brought about by an increased recruitment of immune cells to the site of inflammation and a specific pattern of gene expression in whole blood that may be functionally associated to a swift return to an immune-protective homeostatic state.

RESULTS

Blood cellularity and weight changes in environmentally enriched mice

Over the course of the 14-day enrichment period animals in the EE group consistently demonstrated a significantly lower weight gain compared to those from the standard environment (SE) (**Figure 2A**). Both groups maintained the same intake of food (average 27g per group, per day) while enriched animals trended towards a slightly higher water intake (average 29ml versus 35ml, per day, respectively) (**Figure 2B**).

Peripheral analysis of blood samples taken at day 14 identified no difference in overall numbers of blood leukocytes or individual leukocyte populations between the two groups (**Figure 2C, left panel**). However, further assessment revealed a significant difference in the relative percentages of circulating cell types, with the blood of enriched mice consistently showing a higher percentage of neutrophils and a correspondingly lower percentage of lymphocytes compared to the standard group (**Figure 2D, second and third panels, respectively**). No differences were found in relative levels of monocytes (**Figure 2C, right panel**).

Zymosan induced peritonitis in environmentally enriched mice

We initially investigated differences in immune response between EE and SE animals using a zymosan-induced peritonitis, an experimental model of inflammation that allows the characterization of leukocyte egress into the peritoneal cavity. Six hours after zymosan injection, a significantly higher number of cells was found in the peritoneal lavage of enriched animals (**Figure 3A**). FACS analysis identified the dominant cell population in both groups to be GR-1^{hi}/F4/80^{-}, ascribed as neutrophils (**Figure 3B**). Smaller populations of Gr-1^{hi}/Ly6B.2^{+} cells, attributed as monocytes

were also identified (**Figure 3C**). Across both populations a significantly higher number of cells were present in the cavity of enriched animals compared to those housed in a standard environment (scatter plots in **Figure 3B** and **C**). Analysis of the collected peritoneal lavage fluids identified heightened levels of classical pro-inflammatory cytokines IL-6, KC, MCP-1 and TNF-α in the peritoneal cavity of mice from both groups, however no significant difference was found in relative levels (**Figure 4**).

At 24h post-zymosan, EE animals still possessed a significantly higher number of peritoneal cells compared to those from a standard environment (**Figure 5A**). As with the 6h time point, FACS analysis identified higher numbers of GR-1^{hi}/ F4/80^{-} neutrophils and Gr-1^{hi}/Ly6B.2^{+} monocytes in the enriched group (**Figure 5B** and **C**, respectively). An additional F4/80^{hi}/Gr-1^{-} population, identified as monocyte derived macrophages was also found in both groups. Again numbers of this population were significantly higher in enriched animals (**Figure 5C**). Consistent with previous studies, analysis of the extracted PLF identified pro-inflammatory cytokines levels has fallen greatly in both groups [20] with no difference in relative levels of expression (**Figure 6**).

Effects of ceacal ligation and puncture on environmentally enriched mice

To assess the scale of the EE effect in a further model of peritoneal disease, we subjected EE and SE mice to caecal ligation and puncture (CLP) to produce sepsis. Twelve hours following the CLP protocol a count of the extracted lavage fluids presented a significantly higher number of cells in the peritoneum of enriched mice compared to those from the standard environment (**Figure 7A**). FACS analysis attributed this increase in cell numbers to GR-1^{hi} F4/80^{-} neutrophils (**Figure 7B**).

Bacterial colony counts of the peritoneal fluids showed a trend (non-significant) for a lower overall bacterial count in the peritoneal cavity of enriched animals (**Figure 7B**). Conversely, colony counts in blood were significantly lower in enriched compared to standard mice, suggesting EE animals have a reduced systemic bacterial load compared to mice from a standard environment. As with the peritonitis model, analysis of extracted cell-free peritoneal exudates (**Figure 8**) and serum (data not shown) identified heightened levels of pro-inflammatory cytokines in both groups, however no significant difference was found in relative levels.

Enriched animals present heightened leukocyte activity ex vivo

To further investigate the altered immune response exhibited by enriched animals we subjected isolated leukocytes subsets from SE and EE mice to specific activity assays. Biogel elicited macrophages isolated from the peritoneal cavity of EE and SE and animals had their capacity for phagocytosis assessed by culture with BODIPY-conjugated *E.coli* (dried). Macrophages isolated from enriched mice were found to phagocytose a significantly higher quantity of bacteria over the course of the one hour incubation period than those from animals housed in a standard lab enclosure (**Figure 9A)**. Additionally, the activation status of peripheral blood neutrophils was assessed by stimulation with platelet activating factor (PAF) or Tumour necrosis factor alpha (TNF-α) for 15 or 30 minutes. Following stimulation, cell populations were analysed for the expression of activation markers CD11b and CD62L. A quantitative increase in the former and decrease of the latter (shedding) is considered indicative of a state of activation [21]. No significant differences (calculated by 2-way ANNOVA) were identified in the relative change of expression between both groups (**Figure 9B and C**).

EE mice present a significantly altered genetic profile

The functional association between EE and the efficiency of the inflammatory response brought us to identify underlying mechanisms responsible for this immune-modulating effect. To this end, we took a hypothesis-generating approach, comparing the genetic fingerprints of whole blood taken from enriched and standard environment animals, subject to no inflammatory insult. Using fold change (FC) (>2 or <0.5) and non-adjusted p-value <0.05, of the 34 760 probes present on the chip, 8 genes were identified as upregulated and 5 genes downregulated (**Figure 10A**). Of these genes four of particular interest (s100a8, s100a9, chil 1, sirpb1a) were selected and their heightened expression confirmed via RT-PCR. A significantly higher level of gene expression (relative to housekeeping gene GADPH) was identified in enriched animals compared to those from a standard environment (**Figure 10B**).

To further confirm the gene signature of enriched animals at protein levels, we measured the protein products S100A8 and S100A9 – calgranulin A and B in the PFL (**Figure 11A**), blood plasma (**Figure 11B**) or total cell lysate (**Figure 11C**) of EE or control animals subject to no inflammatory insult. Consistent with the RT-PCR data, we observed significant higher levels of these two soluble mediators in enriched animals compared to standard.

Discussion

This study sought to assess whether defined enhancements to an animal's housing conditions could have a modulating effect on its immune status. We have used this experimental system to model the effects of external environmental factors on the inflammatory response and to investigate the possible underpinning cellular and molecular mechanisms behind these effects.

An enriched environment is known to have a beneficial effect on a variety of neurological parameters. Such research has revealed EE is able to facilitate repair of neurological damage caused by chemical exposure [14], physical trauma [15] and mitigate neurodegenerative disease [16]. Alongside these neurological effects a small number of papers [13,17,19,22-24] have investigated the influence EE has on specific immune parameters, with some suggesting it may have an immune-enhancing effect in response to viral disease[17].

Our results show that environmental enrichment invokes a significant and consistent alteration in the immune repertoire of male CD1 mice: increasing circulating innate immune cells, enhancing macrophage activity and altering the host's gene expression profile to improve bacterial clearance in a model of polymicrobial sepsis. These alterations are evident after only two weeks of enrichment, a time frame previously demonstrated to induce significant physiological effects in neurological research [25] and recently described in our previous study focusing on T cells [26].

We report that EE significantly boosts the cellular response in a model of zymosan induced peritonitis (ZIP). Intraperitoneal injection of zymosan induces a rapid accumulation of PMNs into the peritoneal cavity. This immune response presents a consistent time course, made up of an initial influx of neutrophils 2-10 hours post

injection followed by the later arrival of monocytes and monocyte-derived macrophages at 15-25 hours [20].

Differential analysis of the cell populations in EE revealed elevated numbers of neutrophils and monocytes compared to SE animals at six hours after challenge. Correspondingly, significantly higher numbers of peritoneal macrophages were found in enriched animals 24 hours post injection. The initially higher influx of neutrophils into the EE animal mirrored the higher proportion we observed in circulation in basal conditions. In addition, the discovery of elevated numbers of macrophages and monocytes in the peritoneal cavity suggests an increased speed of egress to the site of inflammation. Lending support to this hypothesis, a previous study which subjected mice to enrichment for up to 18 weeks revealed isolated leukocytes to exhibit heightened proliferation and chemotaxis indicating a common mode of effect for enrichment [13].

The results obtained from ZIP indicate that EE animals are able to mount a more effective inflammatory response i.e. a response to the insult featured by increased clearance of the pathogenic stimuli (through increased recruitment of inflammatory cells) and a measured physiological production of inflammatory cytokines comparative to SE animals. Suggesting that this heightened cellular response may not confer a corresponding increase in tissue damage in EE animals. To confirm this hypothesis we applied a more clinically representative model of inflammation based on live bacterial infection; the ceacal ligation and puncture (CLP) model of polymicropbial sepsis, which provides a much stronger insult able to elicit a marked local (peritoneal cavity) and systemic reaction [27]. Our results show that EE animals are significantly more effective at clearing circulating bacteria than mice from a standard environment and this was again associated with an increase in the number

of peritoneal leukocytes. Combined with the ZIP data, increased numbers of peritoneal leukocytes are a common feature of the EE-evoked cellular response.

The results obtained from these two experimental systems; ZIP and CLP, lead us to hypothesise that changes in external conditions are specifically 'translated' into changes in the cellularity of the inflammatory response ultimately leading to a better containment within the local tissue (in this case peritoneum) and hence a reduced transmission of microbes into the blood stream. It is interesting to note that previous studies have shown that diminished innate leukocyte activity and/or numbers are associated with a high mortality rate in sepsis [28,29], consequent to immune paralysis and an overall failing of the innate response. Congruently, administration of the neutrophil attractant CXCL1 and CXCL2 into the peritoneal cavity after inducing polymicrobial sepsis enhances both neutrophil recruitment and clearance of bacteria, as well as improving host survival [30]. Similarly our recent study [25] on the influence of the enriched environment on T cell response ex-vivo suggest that these cells might contribute to the beneficial effects of this experimental paradigm. Further studies will be needed to dissect the role of the innate and adaptive arms of the immune system in regulating the host immune response.

Interested to further expound the influence of enrichment on the immune system we carried out microarray analysis of RNA extracted from whole blood of EE and SE animals. Most of the genes identified by the array have specific metabolic or immune regulatory function. Of these, four stood out for their role in inflammation: Chil1 (Chitinase-3-like protein 1, chi3l1), Signal-regulatory protein beta 1A (srbp1a), s100a8 and s100a9. Most interestingly, all these genes are endowed with complex modulatory properties both promoting an efficient inflammatory response as well as contributing to host protection.

Chil3L1 is serum protein predominately released by; activated macrophages, neutrophils, endothelial cells and astrocytes**.** Increased circulating levels of Chil3L1 have been detected in patients suffering rheumatoid arthritis [31], sepsis [32] and inflammatory bowel disease [33], suggesting it plays a role in mediating inflammation. Supporting this CHI3L1 secretion is increased by leukocytes upon stimulation by inflammatory cytokines. Interestingly CHI3L1 has also been shown to downregulate cellular responses to the same cytokines [31], indicating that CHI3L1 may be part of a feedback control mechanism regulating against an excessive inflammatory response. While the exact physiological role of Chil3L1 is under ongoing research studies have demonstrated its involvement in a range of immunological processes, including; apoptosis, inflammasome activation, M2 macrophage differentiation, transforming growth factor β1 (TGF-β1) release, dendritic cell activation, and mitogen-activated protein kinase (MAPK) and Akt signalling [34].

Also up-regulated was sirpb1a, a gene coding Signal-regulatory protein beta 1A a glycoprotein receptor expressed on human monocytes and granulocytes [35]. Stimulation of SIRPB1A on murine peritoneal macrophages has been shown upregulate phagocytosis [36] suggesting one possible mechanism for the upregulated macrophage we observed in this study. This activity has in turn been shown to play a protective role in the pathogenesis of Alzeimer's disease, upregulating clearance of amyloid beta aggregates through stimulation of phagocytosis by microglial cells [37]. Further studies revealed that SIRPB1A binding upregulates neutrophil transendothelial migration [38], suggesting one possible explanation for the heightened leukocyte influx we observed in our CLP and ZIP models.

S100A8 and S100A9 together form the protein complex calprotectin which is highly abundant in myeloid cells, with neutrophils expressing highest content (calculated to

290 be ~30% of total cytosolic proteins). We elected to focus on this protein complex for
291 protein confirmation in light of the commercial availability of reliable antibodies and
292 because of its current interest to clinical research. Much of this research focuses on
293 calprotectin's role as a biomarker for inflammatory disease, most especially in
294 intestinal inflammation [39] and more recently neonatal sepsis [40]. Released by
295 neutrophils, calprotectin exerts a direct antimicrobial action by sequestration of
296 essential bacterial nutrients manganese and zinc [41]. The individual proteins have
297 also been found to act as powerful regulators of the innate immune response
298 promoting neutrophil and macrophage accumulation as well as cytokine production
299 [42 43]. Pharmacological administration of S100A8 to mice at the onset of a model of
300 endotoxemia has been reported to significantly increase survival rate [44]. Conversely,
301 elevated calprotectin levels may contribute to early bacterial dissemination and liver
302 injury in a model of *Escherichia coli*-induced sepsis [45].

303 A further observation of this genetic analysis is that the spread of data obtained for
304 EE animals is notably greater than that of SE. This variation may be attributed in part
305 to the fact that CD1 mice are an outbred strain and that hence individual animals will
306 possess an inherent variation in levels of gene expression. Another possibility is that
307 that environmental enrichment itself leads to greater within-group variation.

308 This hypothesis has been addressed in several previous studies with some authors
309 suggesting the level of variation it causes to be negligible [46] while others posit it
310 leads to higher coefficients of variation in many readouts [47]. One common reason
311 cited for this variation is that enrichment leads to a greater level of inter-group
312 fighting probably due to increased territorial dispute and dominance displays [48,49].
313 Further studies would be needed to test the hypothesis that social dominance
314 influence gene expression variability in our experimental settings.

A great deal of research from our lab and many other groups has been invested in exploring mechanisms that favour or contribute to a more effective inflammatory response and a faster re-establishment of beneficial homeostasis. The results of this study provide a new outlook at the possible ways by which the immune response is able to re-establish a state of equilibrium providing a mechanistic underscore to the impact of well-being reproduced herein with the EE exposure. With parallels to the established field of resolution of inflammation where lipids, short peptides or gases have each been described as improving the outcome of an inflammatory response [50], we have revealed another strategy to achieve the same objective. According to this paradigm, the increased cellular response of the host might offer the opportunity to more efficiently respond to an immune insult while mitigating the damage of the inflammatory response. We also identified a specific network of signalling pathways associated with an immune-protective phenotype that could be potentially used to design alternative therapeutic strategies to improve clinical outcome in the context of uncontrolled inflammation.

While environmental enrichment appears to have a tangible effect on the immune response, deeper analysis is required to more fully understand if its effects could be of benefit in the context of disease and more specially disease recovery. Most importantly for the progress of this field, efforts should be made to correlate and stratify clinical and animal studies with one another; only that way will a practicable benefit be derived from this research. EE has already been demonstrated to improve wound rodent wound healing in response to physical trauma [15] and comparable clinical studies have revealed interventions designed to improve the mental

wellbeing of patients leads to a marked improvement in overall health [51] and reduced expression of genes associated with chronic systemic and cellular inflammation [52].

In this study we aimed to further understand how the immune system as whole specifically responds to changes in environmental conditions. The changes we observed at both molecular and cellular levels provide a link between enriched living conditions and an enriched immune response i.e. an improved capability for the host to deal with inflammatory challenges.

These results are in line with a growing body of recent literature that has clearly demonstrated the need to investigate immune disorders and responses as complex multifactorial systems. These should include different factors such as living conditions and psycho-social status [53].

Perhaps the obvious question these findings raise is whether environmental enrichment could serve as a viable treatment for human immune disease or as a prophylactic against microbial or inflammatory disease? The straight answer to this question: is currently a “No”. The results we have obtained cannot be directly translated from mice to humans. What can be learned from these data is that enriching the living conditions of experimental animals modulates the expression of a specific set of immune genes in a host promoting an improved inflammatory response. Providing that we can verify this data in humans, one might us such genes to identify and stratify patients at risk of developing septicaemia or other inflammatory pathologies. A far-reaching ambition for this study would be to pharmacologically modulate the immune genes we have identified to therapeutically treat high-risk patients.

Perhaps the most convincing conclusions to emerge from this study is that we cannot consider biological systems such like experimental animals or indeed human beings in all their diversity as 'fixed' in their immune response. Both mice and humans respond pro-actively to changes in their living conditions. When we refer to pro-actively we mean that both mice and humans change their metabolism, behaviour, blood pressure, tissue regeneration and so and so forth according to changes in their living conditions. Such dynamic adjustments to an altering external and internal environment are often termed as allostasis [54].

The ultimate purpose of animal experimentation is to emulate human physiological responses as accurately as possible. With the results of this study and other in mind, we suggest that a consideration of the environment we house such animals in [55] and its parallels to human living conditions [53] should be an important part of experimental design.

METHODS

Environmental Enrichment (EE) protocol

Upon receipt, male CD1 mice (Charles River UK ltd) aged 6 weeks were placed in groups of six and given one week to acclimatise to the environment of the animal unit. During this time all groups were housed in a standard mouse enclosure consisting of 36x20x14cm (523 sq cm) cage (Allentown) woodchip bedding and nesting material. Following this period, the groups were then re-housed in either the same standard enclosure set up (new cage, bedding and nesting material) or in an enriched enclosure. Enrichment enclosures were 50x38x21cm (1355 sq cm,

Allentown) and included: one wheel, one nest house, one tunnel, ample nesting material, woodchip bedding to a 5cm depth.

All animal enclosures were cleaned out once a week. During cleaning mice were placed in a second enclosure along with all of their enrichment items as well as bedding. Roughly 20% of soiled bedding was left in the enrichment cage. After cleaning all apparatus were replaced exactly as found. Prior to the onset of cleaning mice were allowed a period of roughly 10 minutes to acclimatise to the presence of the researcher. No other researchers were allowed to handle the mice.

Zymosan induced peritonitis (ZIP)

Peritonitis was induced by intraperitoneal (i.p.) administration of 0.5mg of zymosan-A (Sigma-Aldrich) (16mg/kg) diluted in sterile PBS. Six or 24 hours post injection mice were deeply anesthetized by isoflurane inhalation and blood collected by cardiac puncture (anticoagulant: 3% solution of sodium citrate; approximately 1:10 ratio with blood). Immediately following blood extraction animals were sacrificed by CO_2 asphyxiation and a peritoneal lavage performed with 2ml of cold PBS (4°C) containing 3mM EDTA. Peritoneal lavage fluid (PLF) were used to measure cytokine levels using a Bio-Plex mouse cytokine assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's directions. Measurements were carried out using a Luminex Bio-Plex 200 system (Bio-Rad) and then analysed with Bio-Plex Manager 6.1 software (Bio-Rad).

Blood cellular and cytokine profiling

Isolated blood was aliquoted and subject to haematological analysis using a IDEXX ProCyte Dx® Haematology Analyser (IDEXX Laboratories). Plasma was obtained from a second blood aliquot set by centrifugation at 10,000×g for 3 min at 4°C.

Concentrations of IL-6, MCP-1, TNF-α, MIP-2, IFN-γ, S100A8 and S100A9 were determined using a Bio-Plex mouse cytokine assay (Bio-Rad) as reported above.

Flow cytometric leukocyte profiling

Extracted PLF was centrifuged (1500rpm, 5 mins, 4°C) and the pelleted leukocytes re-suspended in 1ml of PBS. Following quantification by haemocytometer, leukocytes were washed and then stained in 100µl of FACS buffer (PBS containing 5% FCS and 0.02% NaN_2) containing CD16/CD32 FcγIIR blocking antibody (clone 93; eBioscience, Wembley, UK) for 30 min at 4ºC. Thereafter cells were stained with the following FITC or PE-conjugated antibodies (eBioscience, Wembley, UK): GR-1 (clone RB6-8C5), F4/80 (clone BM8) and Ly6B.2 (clone 7/4). Cells were labelled with the appropriate concentration of conjugated antibodies for 40 minutes at 4 °C then washed and analysed. In all experiments, stained cells were acquired with an LSR FORTESSA flow cytometer (Becton Dickinson) post hoc analysis was carried out using FlowJo™ 7.0 software (Tree Star, Ashland, OR, USA, Oregon Corporation).

Experimental Sepsis by caecal ligation and puncture (CLP)

Mice were deeply anaesthetised by i.p injection of a 1:1 solution of ketamine (75mg/kg; Vetoquinol) and xylazine (15mg/kg; Bayer). A midline laparotomy was performed to expose the cecum and adjoining intestine. The cecum was then ligated using 4.0 silk sutures (Prolene, Ethicon) just below the ileo-cecal valve and then punctured twice With a 15 gauge needle. Gentle pressure was applied to the cecum in order to extrude a small quantity of faecal matter from the sites of puncture. The cecum was then returned to the peritoneal cavity and the peritoneum and skin sutured closed. Mice were administered resuscitation fluid and allowed to recover from anaesthesia upon a heat pad then returned to their cage. 12 hours following

434 animals was sacrificed by terminal anaesthesia and subject to cardiac puncture,
435 peritoneal lavage and cellular analysis as previously described. Isolated blood and
436 PLF was streaked across nutrient broth (NB) agar plates (10cm diameter) at varying
437 dilutions (10^{-1}–10^{-4}). The plates were incubated overnight following which a count of
438 the bacterial colonies growing on each plate was carried out. Colony forming units
439 per ml (cfu/ml) was calculated by multiplying the number of colonies counted by their
440 dilution factor and dividing this figure by the volume of culture plate. PFL cytokine
441 levels were determined as detailed before.

442

443 All surgeries were conducted using aseptic techniques which meet at least the
444 standards set out in the HO Minimum Standards for Aseptic Surgery. Consequently,
445 post-operative infections were not expected. Any animal showing swelling, redness
446 or reluctance to move, vocalisation when handled or posture suggestive of
447 abdominal pain were killed or referred to the NVS. Systemic analgesic was avoided
448 because it might interfere with the inflammatory/immune reaction (for instance both
449 innate and adaptive immune cells express receptors for opioid that are know to have
450 a modulatory effect on these cells). Treatment was instituted on the advice of the
451 NVS. The animals were be killed by a Schedule 1 method if no improvement is seen
452 in the first 12 hours of treatment.

453

454 **Microarray analysis**

455 Total RNA was extracted from blood of enriched (n=3), and control (n=3) mice using
456 RNeasy® Protect Animal Blood System (Qiagen®, West Sussex, UK). Total RNA
457 was hybridized to Affymetrix Mouse Gene 1.0 ST array chips at UCL Genomics

(London, UK) with standard Affymetrix protocols, using GeneChip Fluidics Station 450, and scanned using the Affymetrix GeneChip Scanner (Affymetrix, Santa Clara, CA, USA). Data were normalized by *rma* of the Bioconductor package, *affy*. Relevant genes were filtered by excluding those without an Entrez ID and those with low expression levels less than 100 by non-logged value. T-statistics were applied across the data set using the Bioconductor package Limma, and differentially expressed genes were identified by $p<0.05$ (non-adjusted p-value) and fold change > 2. Microarray data was approved and uploaded to the GEO database assentation number GSE94279

Real-time polymerase chain reaction

Total RNA was extracted from blood (n=12 for each mouse group) using an RNeasy® Protect Animal Blood System (Qiagen®) according to the manufacturer's protocol. Extracted RNA was reverse transcribed using 2 µg oligo (dT)15 primer, 10U AMV reverse transcriptase, 40U RNase inhibitor (all from Promega Corporation, Madison, WI, USA) and 1.25 mM each dNTP (Bioline, London, UK) for 45 min at 42 °C. The real-time polymerase chain reaction was carried out by using power SYBR GREEN master mix (Thermofisher scientific) and QuantiTect primers (Qiagen). Cycling conditions were set according to the manufacturer's instructions. The sequence-specific fluorescent signal was detected by 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, Cheshire, UK). C_T values for each gene were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then used to calculate expression levels. We used the comparative Ct method to measure the gene transcription in samples. Results are expressed as relative units based on calculation of $2^{-\Delta\Delta Ct}$, which gives the relative amount of a particular gene normalized to an endogenous control (GAPDH).

Neutrophil Activation

Activation assays and analysis were carried out using the same protocols stipulated in [21]. Briefly, blood samples were separated into five aliquots and treated respectively with TNF-α (50 ng/ml), Platelet activating factor (PAF) (10^{-9M}) for 15 or 30 minutes at 37°C, or left unstimulated. Samples were washed and stained for the presence of GR-1 APC (RB6-8C5 clone), CDllb FITC (M1/M70 clone) and L-Selectin (CD62L) PE (Mel-14 clone) on ice for 45 minutes (all antibodies were purchased from, ebioscience). Each sample was lysed and fixed using an Immuno-lyze™ reagent kit (Beckman Coulter). Neutrophils were gated according to forward/side scatter characteristics and positive expression of Gr-1 and CD11b. CD11b expression was recorded as units of fluorescence where the median fluorescence intensity for 10,000 cells was measured in the FL1 green channel (548 nm). In the case of CD11b antibody, the red FL2 channel was used (590 nm). Samples were analysed by a BD LSRFortessa II™ with post hoc analysis carried out using FlowJoTM 7.0 software (Tree Star, Ashland, OR, USA, Oregon Corporation).

Phagocyte Function

The following procedure was based on that of [56]. Animals were injected i.p with 0.5ml of a 4% solution of biogel P-100 (Bio-Rad) and left undisturbed for four days. Animals were then sacrificed and subject to peritoneal lavage with 10ml of PBS. Collected cells were washed twice then re-suspended in complete RPMI-1640 media (Sigma-Aldrich). Isolated cells were confirmed as macrophages via microscopic analysis then counted and seeded at $0.2x10^6$ cells per well in 200µl of warm complete RMPI on a 96 well, black walled, clear bottomed plate (Grenier one) and allowed to adhere for two hours at 37˚C (5% CO2). Macrophages were then

stimulated with BODIPY 576/589 (Invitrogen) conjugated, lyophilised *E.coli* (sigma) added at a final concentration of 1mg/ml to each well. Following one-hour cell activity was halted and any non-phagocytosed *E.coli* removed by washing three times with cold PBS. Fluorescence was read at 570 and 590nm using a multiscan FC plate reader.

Statistical Methods

According to the nature of the data obtained a t-test (two-tailed), or ANOVA (one or two-way) was performed. Time course observations were analysed with multiple t-tests and the Holm-Sidak post-hoc test. Behavioural data was analysed via non parametric analysis using the Mann Whitney U test. All statistical analysis was performed using GraphPad PRISM software V6.0 (Graphpad inc) with the exception of microarray analysis which was carried out as stated in 2.13.3 using the software package LIMMA (Bioconductor). Data was analysed for normality using the D'Agostino & Pearson omnibus normality test.

Declaration of Study Approval

Adult male CD1 mice (5 Weeks old) obtained from Charles River UK ltd were used for all experiments. Animals were kept under standard conditions: in individually ventilated enclosures, food and water provided *ad libitum*, 12-hour light-dark cycle. All animals were allowed a seven-day acclimatisation period before any experimental procedure was performed upon them. All experiments were approved and performed under the guidelines of the Ethical Committee for the Use of Animals, Bart's and The London School of Medicine and Home Office Regulations [57]. PPL 80/8714

Author Contributions

S.B Designed research studies, conducted experiments, acquired and analyzed data and wrote the manuscript.

B.G and T.G. Provided technical assistance throughout the CLP studies.

M.O analysis of acquired microarray data.

M.P Advice on experimental design and editing of the manuscript

F.D Designed of research studies assistance conducting experiments.

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Figures

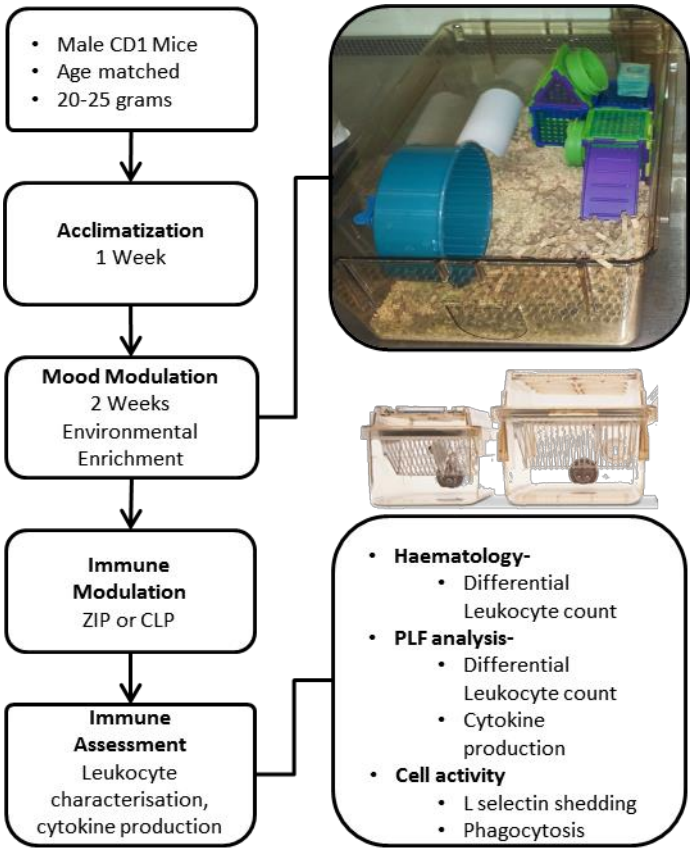


Figure 1. Experimental design, time line and depiction of the enriched cage setup. Enrichment conditions were kept consistent for all experiments. ZIP – Zymosan induced peritonitis, CLP- Caecal ligation and puncture

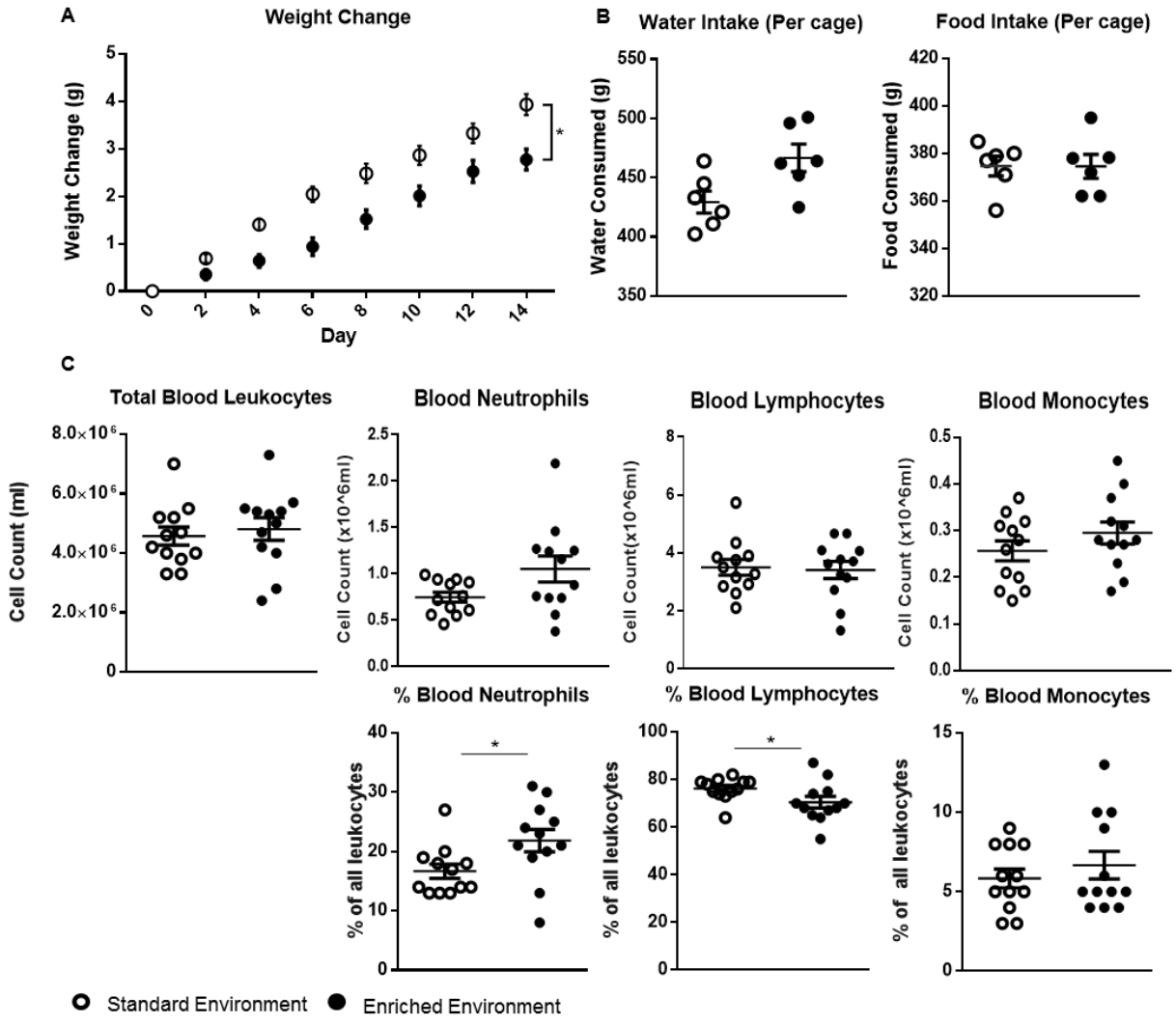


Figure 2. Altered metabolic and blood parameters in enriched mice. **A** (left to right) Environmentally enriched (EE) animals present a reduced rate of weight gain a two week period when compared to those housed in a standard lab environment (SE), while maintaining similar average food and water intake **B**. **C** Total numbers of circulating leukocytes and relative percentages of leukocyte populations in the blood of EE and SE mice. Values are expressed as mean ±s.e.m. of 12 mice per group and are representative of n=3 independent experiments with similar results. Statistical significance determined via t test $P < 0.05$.

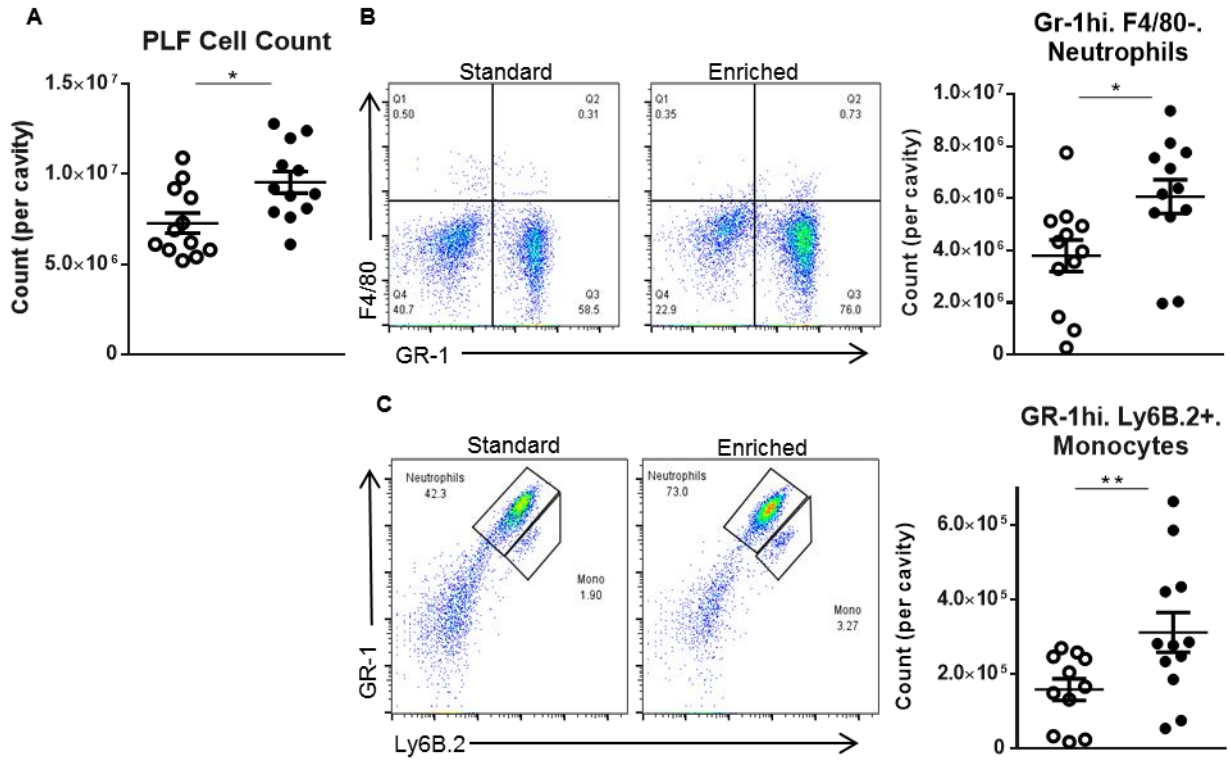
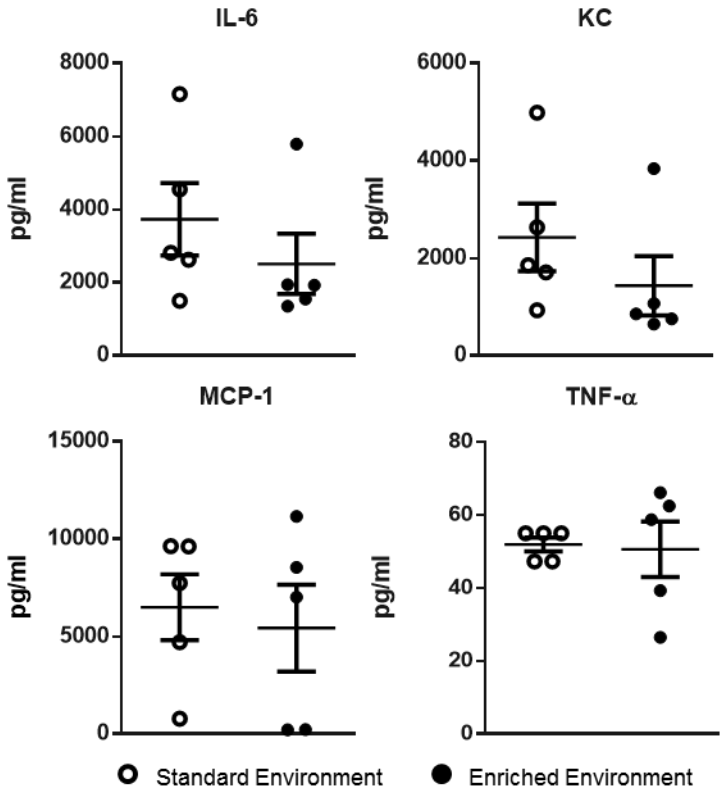


Figure 3. Increased peritoneal leukocytes in enriched mice following six hours of zymosan induced peritonitis. A Peritoneal cell count of SE and EE animals**. B** Representative FACS profiles of F4/80 vs. GR-1 expressing cells and comparative plots of total peritoneal populations of GR-1hi F4/80- neutrophils in SE and EE mice. **C** Representative FACS profiles of Gr-1 vs. Ly6b.2 expressing cells and comparative plots of total peritoneal populations of GR-1hi Ly6b.2+ monocytes in SE and EE mice. Values are presented as individual data points mean ±s.e.m. of 12 mice (with the exception of the enriched FACS plots in b & c where one sample was lost) and are representative of n=3 experiments. Statistical significance determined via t test $P < 0.05$.



568

569 **Figure 4. No difference in circulating Inflammatory cytokines in enriched mice following**
570 **six hours of zymosan induced peritonitis**. Comparative expression of innate inflammatory
571 cytokines Interleukin six (IL-6), Keratinocyte chemoattractant (KC), Monocyte chemotactic
572 protein one (MCP-1) and Tumour necrosis factor alpha (TNF-α) in EE and SE animals. Values
573 are presented as individual data points mean ±s.e.m. of 5 mice and are representative of
574 n=3 experiments.

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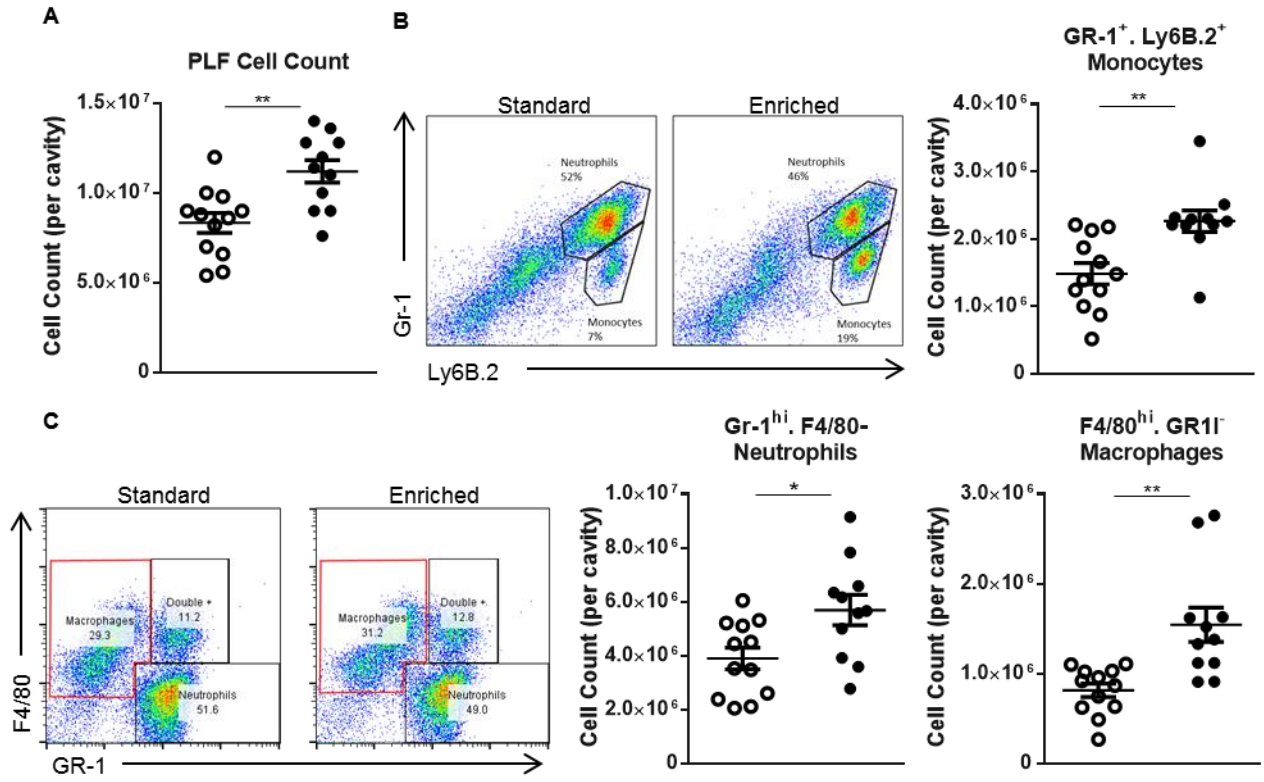
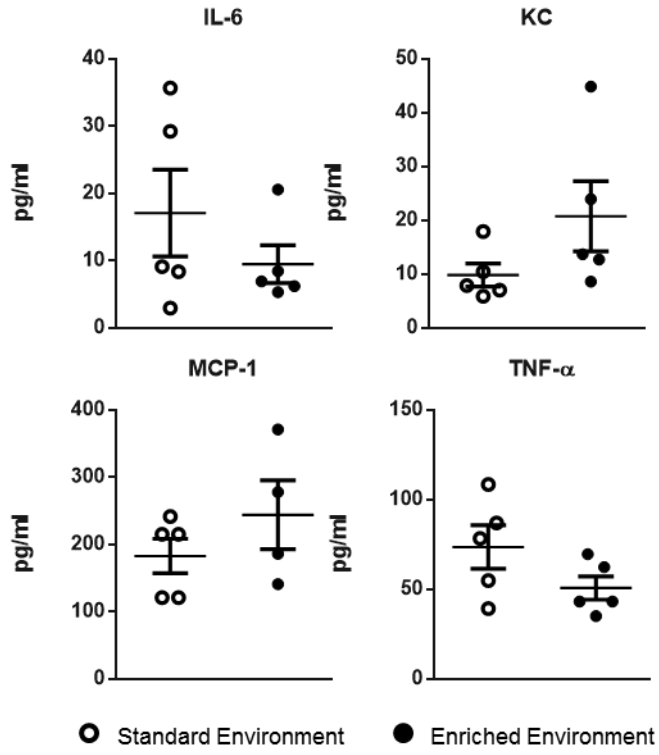


577
578 **Figure 5. Increased peritoneal leukocytes in enriched mice following 24 hours of zymosan**
579 **induced peritonitis**. **A** Peritoneal cell count of standard environment (SE) and enriched
580 environment (EE) animals. **B** Representative FACS profiles of Gr-1 vs. Ly6b.2 expressing cells
581 and comparative plots of total peritoneal populations of GR-1^{hi} F4/80^{-} neutrophils in SE and
582 EE mice. **C** Representative FACS profiles of F4/80 vs. GR-1 expressing cells and comparative
583 plots of total peritoneal populations of GR-1hi Ly6b.2^{+} monocytes and F4/80^{hi} Gr-1^{-}
584 macrophages in SE and EE mice. Values are presented as individual data points mean ±s.e.m.
585 of 12 mice and are representative of *n*=3 experiments. Statistical significance determined via
586 t test $P < 0.05$.
587



588

589 **Figure 6. No difference in circulating Inflammatory cytokines in enriched mice following 24**
590 **hours of zymosan induced peritonitis.** Comparative expression of innate inflammatory
591 cytokines Interleukin six (IL-6), Keratinocyte chemoattractant (KC), Monocyte chemotactic
592 protein one (MCP-1) and Tumour necrosis factor alpha (TNF-α) in standard environment (SE)
593 and environmentally enriched (EE) animals. Values are presented as individual data points
594 mean ±s.e.m. of 5 mice and are representative of n=3 experiments.
595

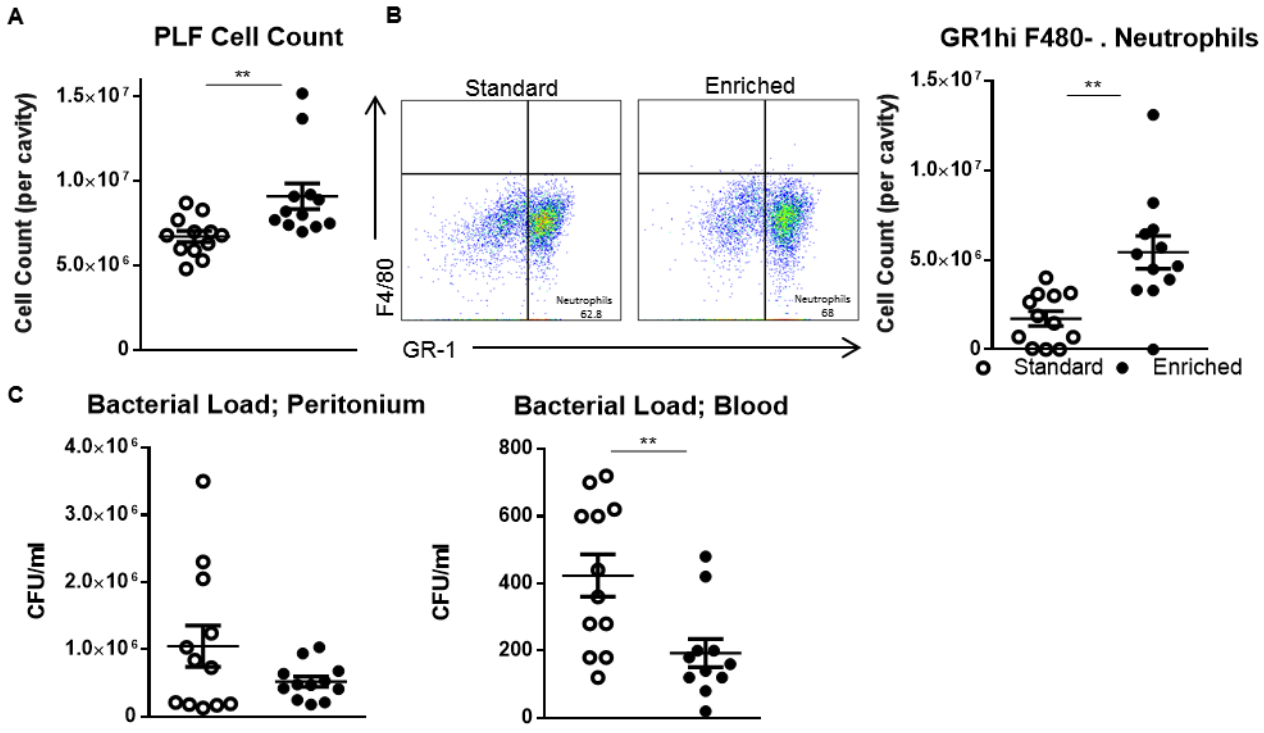
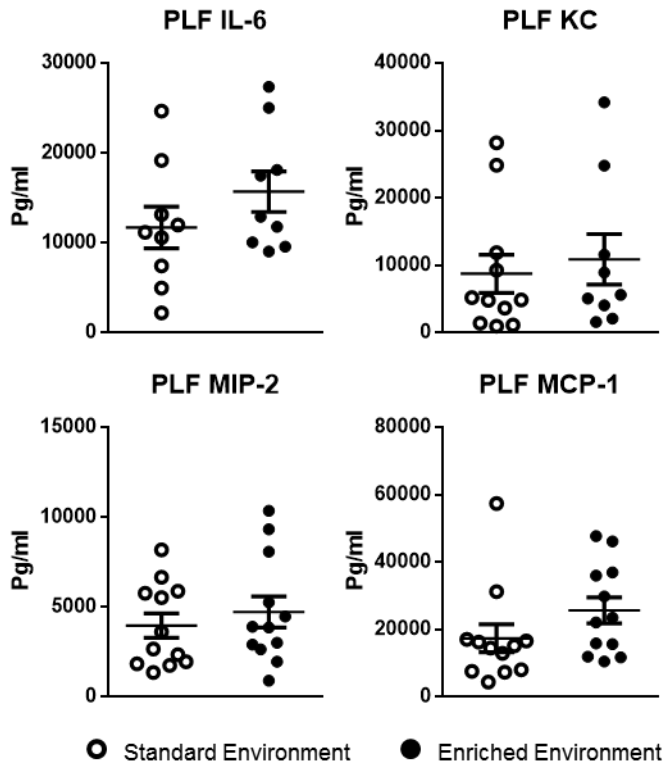


Figure 7. Enriched mice exhibit improved bacterial clearance in a experimental model of sepsis. **A** Peritoneal cell count of standard environment (SE) and environmentally enriched (EE) animals. **B** Representative FACS profiles of F4/80 vs. GR-1 expressing cells and comparative plots of total peritoneal populations of GR-1^{hi} $F4/80^{-}$ neutrophils in SE and EE mice. **C** Respective bacterial colony counts in peritoneal lavage fluid and blood of SE and EE animals values represented a colony forming units per ml. Values are presented as individual data points mean ±s.e.m. of 12 mice and are representative of *n*=2 experiments. Statistical significance determined via t test $P < 0.05$.



607

608 **Figure 8. No difference in circulating Inflammatory cytokines in enriched mice following 12**
609 **hours of sepsis induced by caecal ligation and puncture.** Comparative expression of innate
610 inflammatory cytokines Interleukin six (IL-6), Keratinocyte chemoattractant (KC),
611 Macrophage inflammatory protein two (MIP-2) and Monocyte chemotactic protein one
612 (MCP-1) in standard environment (SE) and environmentally enriched (EE) animals. Values are
613 presented as individual data points mean ±s.e.m. of 12 mice and are representative of n=2
614 experiments.
615

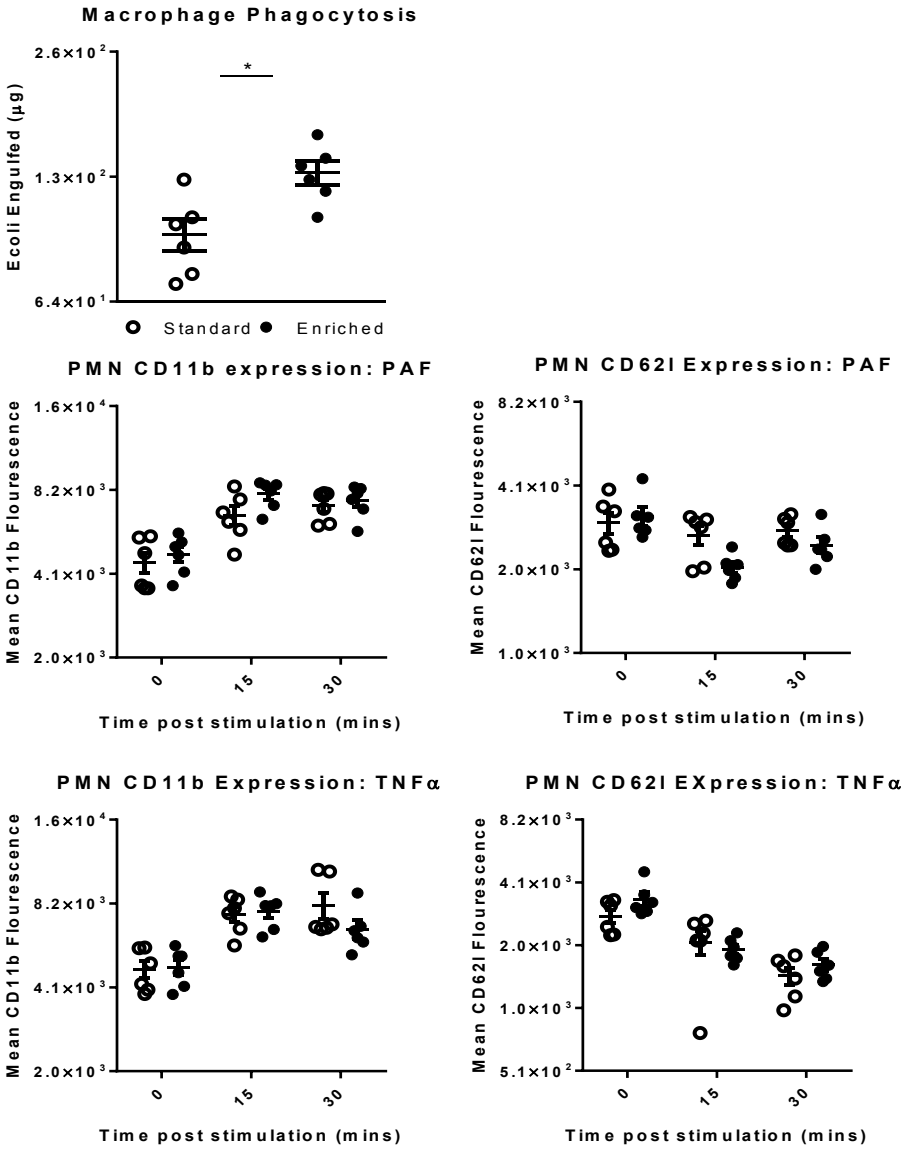


Figure 9. Differential ex vivo activity of leukocytes isolated from mice housed in a standard or enriched lab environment. A Comparative levels of BODIPY linked *E.coli* phagocytosed by bio gel generated macrophages in one hour were measured in mice housed in an standard (SE) or enriched environment (EE). EE macrophages were observed to engulf a significantly higher number of bacterial particles than SE. *p<0.005, t-test. Representative of N=3 experiments, 12 mice per experiment. **B** Analysis of relative levels of PTGS2 and NOS2 gene expression by EE and SE macrophages following overnight stimulation with 0, 1 or 10 ng of LPS revealed a significantly lower level of expression in EE animals and distinct time points. *p<0.005, t-test. Representative of N=1 experiments, 12 mice per experiment. **C** Comparative levels of CD11b and CCD62L expression by activated polymorphonuclear blood leukocytes extracted from EE and SE animals were measured by flow cytometry. Following stimulation with either TNF-α (50 ng/ml), (c), or PAF, (10−9M), (d) for 15 or 30 minutes no differences in expression were identified. Values are presented as individual data points mean ±s.e.m. of six mice N=2 experiments. Significance calculated (none found) by two-way ANOVA ($P < 0.05$).

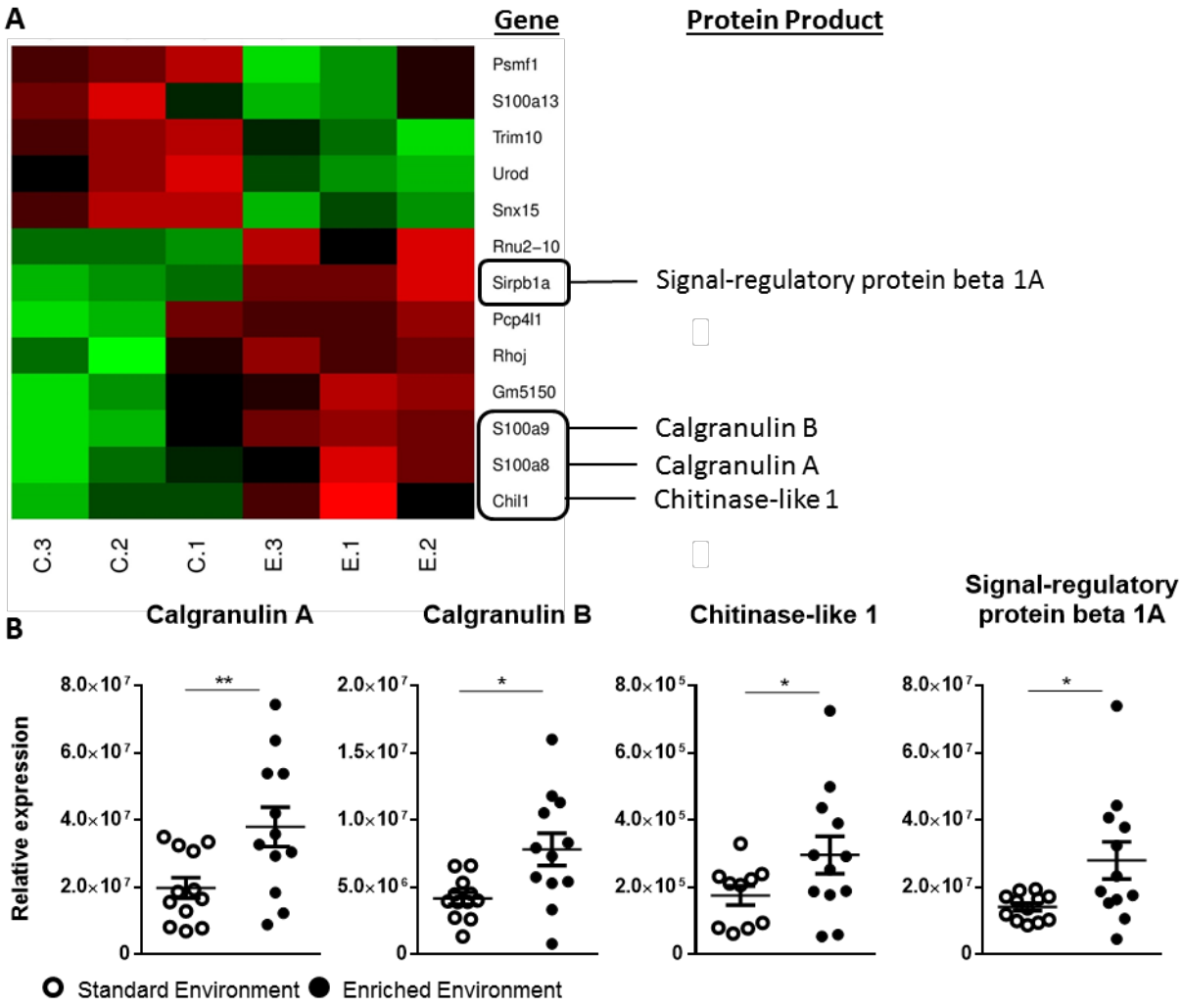


Figure 10. Enriched mice exhibit an altered immuno-regulatory gene profile. A Heatmap analysis of microarray data of blood from standard environment (SE) and environmentally enriched (EE) mice. Hierarchical clustering and heatmap analysis of the filtered genes and the protein products of genes of interest **B** Real time PCR analysis of four genes of interest highlighted in the microarray. Statistical significance determined via t test $P < 0.05$. Values are presented as individual data points mean ±s.e.m. of 12 mice.

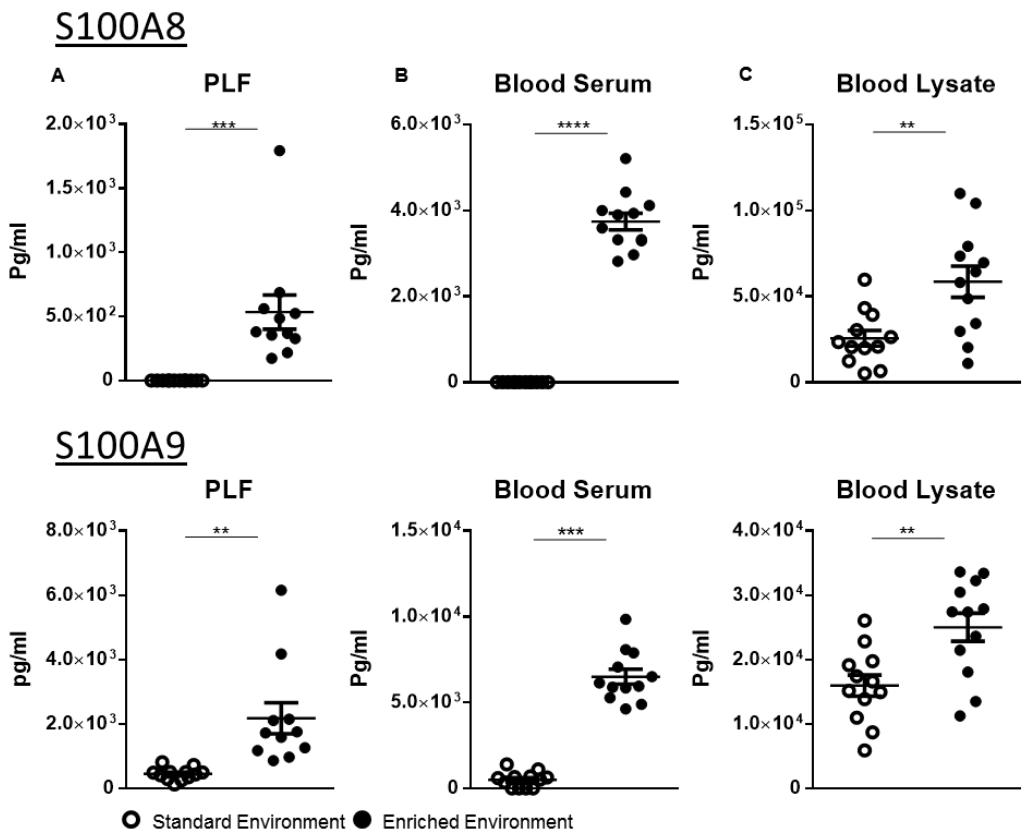


Figure 11. Enriched mice present heightened levels of circulating calgranulin. Comparative expression of the individual diamers of calprotectin (S100A8 & S100A9) in: A Peritoneal Lavage. B Blood serum. C Blood pellet lysate. Samples were taken from enriched (EE) and standard environment mice (SE) subject to no additional immune modulation. Values are presented as individual data points ±s.e.m. of 12 mice from SE and 11 for EE *n*=1 experiments . Significance calculated by T test ($P < 0.05$) .

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